Hiroshima Journal of Medical Sciences
Vol. 30, No. 2, June, 1981
**HIJM** 30-13



# EFFECT OF THE PURIFIED UNSAPONIFIABLE FRACTION OF SOYBEAN ON THE LIPID METABOLISM AND GALLSTONE IN MICE*)

By

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(Received February 9, 1981)

## ABSTRACT

The influences of the purified unsaponifiable fraction of soybean (PUFS) on gallstone formation and dissolution and related lipid metabolism were investigated in mice fed lithogenic diet in comparison with soybean sterols (SBST).

In experiment I, the effect of each drug on gallstone formation was studied during 8 weeks. All of mice fed lithogenic diet had a large number of gallstones in gallbladder but PUFS treatment inhibited markedly the stone production. The influence of SBST alone was weaker than PUFS itself. In this experiment, the serum and liver cholesterol levels were parallel to the degree of gallstone production. The liver enlarged three times as large as control under the lithogenic condition was improved with PUFS or SBST. In experiment II, the effect of drugs on gallstone dissolution was investigated. The mice already having gallstone after feeding a lithogenic diet for 8 weeks were kept on the normal chow with or without PUFS for further 5 weeks. The tendency to dissolve gallstone was not observed in both groups but serum and liver cholesterol contents and liver weight returned more rapidly to normal level in the PUFS treated group. As experiment III, the mice already having gallstone were continuously maintained on the lithogenic diet with or without PUFS during 6 weeks. No effect on gallstone dissolution was found but the desirable influences on lipid metabolism were also clearly observed.

## INTRODUCTION

It is well known that lipids play very important roles in bile and their metabolic disorder is liable to cholelithiasis and that the formation and enlargement of cholesterol gallstone are very closely related with the amounts and ratio of bile lipid components, which in turn are directly or indirectly affected by the lipid metabolism in other organs, but there is no simple relationship between the lipid changes in bile and those in such other tissues as blood, liver,

*) 梶山梧朗，川本敏雄，林　敏広，大木正彦：ラットにおける脂質代謝および胆石に及ぼす大豆純化不鹸化物の効果

gastrointestine and so no.: Recently several reports have revealed that some hypolipidemic agents which clearly decrease the plasma cholesterol level increase reversely cholesterol concentration in bile to aggravate cholelithiasis, which sends out a warning that the clinical means of so-called lipid lowering agents depend on their mode and site of action[1-3].

The purified unsaponifiable fraction of soybean (abbreviated as PUFS in what follows) contains as main components soybean sterols (SBST), tocopherols and unsaturated fatty acids, and its marked hypolipidemic effect has been demonstrated in animal[4-6] and clinical studies[7]. It is now clearly proved that phytosterols which are the principal constituents of PUFS competitively inhibit the intestinal absorption of exogenous and endogenous cholesterol and hence decrease the plasma lipid levels[8]. In addition, as a another aspect of phytosterols in the influences on lipid metabolism, its post absorption effect has been discussed[9-14] and furthermore, many reports have refered to the various influences of tocopherol and unsaturated fatty acid on lipid turnover. So PUFS may regulate the lipid metabolism in many respects. It seems very meaningful, therefore, to study the direct or indirect effects of PUFS on bile stone formation or dissolution. The present communication describes the results of the experiments about the influences of PUFS on cholesterol-induced gallstone and related lipids in mice.

## MATERIALS AND METHODS

### Test drugs

PUFS: The purified unsaponifiable fraction of soybean (PUFS) produced by Ajinomoto Co. Ltd. was used, which contained as main components 41.0% of soybean sterols (SBST), 19.0% tocopherols and 25.6% of unsaturated fatty acids. The latter fraction was mainly consitituted of 48.0% of linoleic acid, 27.2% of oleic acid, 13.7% of palmitic acid and 5.3 % of linolenic acid.

SBST: This was separated from PUFS and contained ca. 45% of β-sitosterol, ca. 30% of stigmasterol and ca. 25% of campesterol.

### Animals and Diets

Four-weeks old CD-1 male mice from Japan Charles River Breeding Laboratory, Japan, were used as experimental animals and cholesterol (CH), cholic acid (CA) and test compounds were admixed with the powered basal diet, CE-II, purchased from Nippon Clea Co. Ltd., The amount of PUFS and SBST to be added was calculated so that the equal amount of soybean sterols was contained in two corresponding groups, i.e. 1.5% PUFS group correspond to 0.6% SBST group and 2.9% and 5.8% PUFS group to 1.2% and 2.4% SBST groups, respectively. Animals had free access to water and diet.

### Animal experiments

1) Exp. I-Prevention of gallstone formation

Mice weighing ca. 14 g were devided into eight groups of each fifteen animals and each group was fed with the experimental diet shown

**Table 1.** Diet composition and experimental period in exp. I

| Group | Diet composition (0 — 8 weeks) |
|---|---|
| I (n=15) | commercial chow (CE-II) |
| II (n=15) | lithogenic diet (CE-II+1.2%CH+0.5%CA) |
| III (n=15) | 〃 〃 +1.5% PUFS |
| IV (n=15) | 〃 〃 +2.9% PUFS |
| V (n=15) | 〃 〃 +5.8% PUFS |
| VI (n=15) | 〃 〃 +0.6% SBST |
| VII (n=15) | 〃 〃 +1.2% SBST |
| VIII (n=15) | 〃 〃 +2.4% SBST |

CH: cholesterol, CA: cholic acid, PUFS: purified unsaponifiable fraction of soybean, SBST: soybean sterols

in Table 1. After feeding for eight weeks and fasting overnight, blood samples were taken from carotid artery, and liver and gallbladder were excised.

2) Exp. II and III-Dissolution of gallstone

In exp. II, ninety six of mice which already had gallstone in gallbladder after feeding lithogenic diet (1.2% CH and 0.5% CA) for eight weeks were devided into two groups of each forty eight animals. One group was kept on the normal diet and another group on the experimental diet containing 2.9% PUFS for further five weeks as shown in Table 2. On 7th, 14th, 21th and 35th day thereafter, plasma, liver and gallbladder were obtained after overnight fast from twelve mice, respectively.

In exp. III, seven mice already had gallstone were continuously kept for six weeks on lithogenic diet alone and other eight mice with gallstone on the lithogenic diet supplemented with 2.9% of PUFS and as a reference, normal group was accompanied as shown in Table 3 and then analytical samples were obtained as in the case of exp. II.

Method for the observation of gallstone and the determination of lipids.

The separated gallbladder was opened on a glass slide and the contents were examined under a low power microscope (×40). Plasma cholesterol was analysed by O. P. A. method (cholesterol assay kit from Wako Chemicals, Tokyo) and plasma phospholipid by phosphorous-molybdic acid method (phospholipid assay kit from Wako Chemicals, Tokyo). Liver lipids were excreted by Forch procedure and its cholesterol and phospholipid were determined by the modified Zak-Henly method and Bartlett method, respectively.

## RESULTS

### Exp. I-Prevention of gallstone formation

Changes in body weight, food intake and liver weight in each group are compiled in Table 4. All animals gained considerable body weight during experiment and supplementation of cholesterol, PUFS and SBST in commercial normal chow had little influences on food intake. Relative liver weight of the normal group I, 0.38 g/10 g body weight, raised to 1.01 g in lithogenic group (group II), indicating the liver enlargement by cholesterol and cholic acid loading and enlarged liver also changed in its colour to be creamy. This hepatomegaly and fatty liver like appearence were markedly inhibited dose dependently by PUFS and SBST as shown in Table 4; i. e. in the case of supplementation of PUFS by 1.5, 2.9 and 5.8 w/w%, relative liver weights were 0.80, 0.56 and 0.50 g/10 g b. w., respectively. Similarly, SBST addition at the levels of 0.6, 1.2 and 2.4% gave the relative liver weight, 0.81, 0.75

**Table 2.** Diet composition and experimental period in exp. II

| Group | Diet composition (0 — 8 — 9 — 10 — 11 — 13 (weeks)) | |
|---|---|---|
| IX (n=48) | lithogenic diet | commercial chow |
| X (n=48) | lithogenic diet | commercial chow+2.9% PUFS |

**Table 3.** Diet composition and experimental period in exp. III

| Group | Diet composition (0 — 8 — 14 (weeks)) | |
|---|---|---|
| XI (n=9) | commercial chow | commercial chow |
| XII (n=7) | lithogenic diet | lithogenic diet |
| XIII (n=8) | lithogenic diet | lithogenic diet+2.9% PUFS |

**Table 4.** Body weight, food intake and liver weight of mice fed gallstone inducing diet in exp. I

| Group | Body weight | | | Food intake | Liver weight | |
|---|---|---|---|---|---|---|
| | initial (g) | final (g) | gain (g) | (g/day/mouse) | whole (g) | relative (g/10 g) |
| I | 14.1 ±0.1 | 41.4[a)] ±1.3 | 27.3 ±1.3 | 6.3 ±0.1 | 1.41[c)] ±0.05 | 0.38[c)] ±0.01 |
| II | 13.9 ±0.3 | 38.8 ±0.7 | 24.4 ±0.8 | 5.1 ±0.3 | 3.43 ±0.13 | 1.01 ±0.04 |
| III | 14.3 ±0.1 | 40.0 ±0.9 | 25.8 ±0.9 | 5.5 ±0.3 | 2.83[a)] ±0.19 | 0.80[b)] ±0.04 |
| IV | 14.5 ±0.1 | 41.1[a)] ±0.8 | 26.7 ±0.8 | 5.8 ±0.1 | 2.02[c)] ±0.08 | 0.56[c)] ±0.02 |
| V | 14.5 ±0.1 | 41.1[a)] ±0.9 | 26.6 ±1.0 | 5.9 ±0.2 | 1.84[c)] ±0.07 | 0.50[c)] ±0.02 |
| VI | 14.4 ±0.1 | 41.3[b)] ±0.7 | 26.9 ±0.8 | 6.0[a)] ±0.1 | 2.95[a)] ±0.17 | 0.81[b)] ±0.04 |
| VII | 14.5 ±0.1 | 39.4 ±0.6 | 24.9 ±0.7 | 6.7[b)] ±0.2 | 2.60[c)] ±0.15 | 0.75[c)] ±0.04 |
| VIII | 14.4 ±0.1 | 41.7[b)] ±1.0 | 27.3 ±1.0 | 6.6[b)] ±0.3 | 2.55[c)] ±0.13 | 0.70[c)] ±0.03 |

Each value represents the mean±S. E..

Statistically significant against control (group II): a) $p<0.05$, b) $p<0.01$, c) $p<0.001$.

Food intake was measured during 28th day to 32nd of the experiment.

and 0.70 g/10 g b. w. which were still smaller than that of control group but much larger than that of the corresponding PUFS groups.

Influences of PUFS and SBST on cholesterol and phospholipid levels in plasma and liver are summarized in Table 5. Plasma cholesterol elevated with a lithogenic diet by 50% from the normal levels was significantly decreased by PUFS and SBST. Influences of PUFS on liver cholesterol contents were still more remarkable. Thus lithogenic diet shifted the liver cholesterol levels from 6.0 mg/g in normal group I to 73.7 mg/g in control group II, which decreased to 59.3, 22.9 and 11.6 mg/g (group III, IV and V) by supplementation of 1.5, 2.9 and 5.8% PUFS, respectively. SBST, too, clearly inhibited the elevation of liver cholesterol level but its extent was weaker than that of the corresponding PUFS group (group IV vs VII and group V vs VIII). Phospholipid levels in plasma and liver were rarely affected by lithogenic condition and drug treatment as seen in Table 5. Incidences of gallstone in various conditions are shown in Table 6. Normal group I had no gallstone but lithogenic diet induced 100% cholesterol cholelith as seen in group II. Generally there were observed in gallbladder several complete stones composed of cholesterol along with many plate crystallines and their aggregates. This high incidence of bile stone was markedly decreased by PUFS, i. e. 1.5% PUFS supplement resulted in 64.3 % of stone formation (group III) which was further depressed to 13.3% by 2.9% of PUFS and 5.8% of this drug completely prevented the production of cholesterol crystalline in bile (group V). The same dose-dependent prevention of gallstone was also recognized by SBST, thus in group VI fed the diet containing 0.6 % of SBST, 80.0% of animals had cholesterol gallstones and group VII with 1.2% SBST and group VIII with 2.4% SBST had 64.3% and 26.7% of stone formation, respectively. These results showed that if the same amount of SBST was contained, PUFS was surely superior to SBST alone (group III vs VI, group IV vs VII and group V vs VIII).

**Exp. II and III–Dissolution of gallstone**

**Table 5.** Lipid levels of serum and liver of mice fed gallstone inducing diet in exp. I

| Group | Serum (mg/dl) | | | Liver (mg/g wet) | | |
|---|---|---|---|---|---|---|
| | CH | | PL | CH | | PL |
| I | 176.3[c] ±8.6 | | 249.7 ±11.5 | 6.0[c] ±0.1 | | 36.2[c] ±0.5 |
| II | 265.7 ±18.7 | | 217.8 ±13.2 | 73.7 ±4.7 | | 32.6 ±0.8 |
| III | 244.9 ±9.3 | (7.8%) | 202.4 ±12.7 | 59.3[a] ±2.3 | (19.5%) | 33.8 ±0.7 |
| IV | 196.9[b] ±7.6 | (25.9%) | 189.3 ±12.0 | 22.9[c] ±2.7 | (68.9%) | 37.1[c] ±0.5 |
| V | 195.7[b] ±7.2 | (26.4%) | 206.9 ±10.2 | 11.6[c] ±1.2 | (84.3%) | 35.9[c] ±0.4 |
| VI | 221.7 ±11.3 | (16.6%) | 172.0[b]* ±6.0 | 58.5[a] ±3.9 | (20.6%) | 33.5 ±2.2 |
| VII | 213.9[a] ±12.1 | (19.5%) | 189.1 ±11.1 | 45.5[c]** ±4.4 | (38.3%) | 36.4[c] ±0.5 |
| VIII | 209.5[a] ±12.9 | (21.1%) | 190.9 ±10.2 | 30.5[c]*** ±4.1 | (58.6%) | 33.3 ±1.2 |

Each value represents the mean±S. E..

Figures in parenthesis show the decrease percentage against control (group II).

Statistically significant against control: a) $p<0.05$, b) $p<0.01$, c) $p<0.001$.

* Statistically significant against group III at $p<0.05$.

** 〃 〃 〃 group IV at $p<0.001$.

*** 〃 〃 〃 group V at $p<0.001$.

**Table 6.** Incidence of gallstone in mice fed gallstone inducing diet in exp. I

| Group | | Incidence of gallstone* | Percentage** |
|---|---|---|---|
| I | Normal | 0/15 | 0 |
| II | Control | 15/15 | 100 |
| III | PUFS 1.5% | 9/14 | 64.3 |
| IV | PUFS 2.9% | 2/15 | 13.3 |
| V | PUFS 5.8% | 0/15 | 0 |
| VI | SBST 0.6% | 12/15 | 80.0 |
| VII | SBST 1.2% | 9/14 | 64.3 |
| VIII | SBST 2.4% | 4/15 | 26.7 |

* numbers of animals with gallstone/numbers of animals used

** gallstone formation percentage

Lipid levels in serum and liver, the relative liver weight and gallstone incidences in exp. II and III are summerized in Table 7 and 8, respectively. In exp. II the elevated cholesterol levels in serum and liver of control group IX spontaneously declined in the course of time after exhanging the lithogenic diet for the normal chow but their declining speed was faster in group X supplemented with 2.9% of PUFS than in group IX without PUFS, though five weeks later lipids levels were almost same in both groups. The same tendency was observed with regard to the relative liver weight, namely, liver enlargement was regressed more rapidly

**Table 7.** Cholesterol levels, liver weight and incidence of gallstone in mice fed curative diet in exp. II

| Curative weeks | Cholesterol concentration serum (mg/dl) | | liver (mg/g wet) | | Liver weight (g/10 g) | | Gallstone | |
|---|---|---|---|---|---|---|---|---|
| | Ⅸ (normal) | X (PUFS) | Ⅸ (normal) | X (PUFS) | Ⅸ (normal) | X (PUFS) | Ⅸ (normal) | X (PUFS) |
| 0 | 265.7 ±18.7 | | 73.7 ±4.7 | | 1.01 ±0.04 | | 15/15 | |
| 1 | 185.7 ±9.6 | 163.0 ±8.5 | 59.6 ±3.2 | 53.8 ±6.7 | 0.81 ±0.06 | 0.72 ±0.05 | 12/12 | 8/12 |
| 2 | 166.7 ±12.9 | 146.8 ±9.6 | 41.5 ±6.4 | 25.0 ±5.0 | 0.67 ±0.06 | 0.59 ±0.03 | 10/12 | 10/12 |
| 3 | 167.0 ±7.8 | 159.3 ±6.8 | 25.4 ±4.6 | 12.1 ±1.7 | 0.61 ±0.08 | 0.54 ±0.02 | 10/12 | 10/12 |
| 5 | 159.0 ±11.8 | 157.2 ±8.6 | 8.9 ±1.7 | 7.8 ±0.2 | 0.50 ±0.04 | 0.46 ±0.01 | 12/12 | 11/12 |

Each value represents the mean±S. E.

Incidence of gallstone represents the ratio of animals with gallstone to numbers of animals used.

Statistically significant against corresponding normal group: $p<0.05$.

by PUFS feeding than by normal diet only.

Gallstones were almost retained intact even at fifth week after the exchange of lithogenic diet for normal chow with or without of PUFS as seen in Table 7. In exp. III, the serum cholesterol risen by the continuous feeding of lithogenic diet for fourteen weeks was decreased by 26.4% through a blend of 2.9% PUFS. The effect of PUFS supplementation on liver cholesterol content was striking; 2.9% of PUFS (group XIII) decreased the liver cholesterol by 53.8% from the control (group XII) level. Phospholipid was rarely affected in both serum and liver by PUFS. Those desirable alteration by PUFS of serum and liver lipid above mentioned did not bring about gallstone dissolution at all within the experimental period as seen in Table 8.

## DISCUSSION

β-sitosterol, a representative hypolipidemic agent to inhibit the intestinal cholesterol absorption, was shown to depress the gallstone

**Table 8.** Lipid levels of serum and liver and incidence of gallstone in mice fed gallstone inducing diet in exp. III

| Group | Serum (mg/dl) | | Liver (mg/g wet) | | Gallstone |
|---|---|---|---|---|---|
| | CH | PL | CH | PL | |
| Ⅺ (normal) | 167.3±9.0[a)] | 256.5±12.2 | 8.4±0.4[c)] | 34.8±0.6 | 0/9 |
| Ⅻ (lithogenic diet) | 273.5±20.0 | 231.8±24.5 | 81.0±3.4 | 32.2±0.6 | 7/7 |
| ⅩⅢ (lithogenic diet+ PUFS 2.9%) | 201.3±16.3[c)] (26.4) | 216.5±15.0 | 37.4±6.8[c)] (53.8) | 37.8±1.6[a)] | 7/8 |

Each value of lipid levels represents the mean±S. E..

Incidence of gallstone represents the ratio of numbers of animals with gallstone to numbers of animals used.

Figures in parenthesis show the decrease percentage compared with group XII.

Statistically significant against group XII: a) $p<0.05$, c) $p<0.001$.

formation in experimental animals[15-17] and further to promote the cholelith dissolution in clinical studies[18-20]. It is reasonable to expect the same effect of PUFS which contains as main components plant sterols including β-sitosterol. From these view points the influence of PUFS on cholelithiasis and lipid metabolism were investigated using mouse model in the present studies.

As mentioned above, all mice fed the lithogenic diet for eight weeks had gallstones in gallbladder which were generally the mixture of a good deal of fine cholesterol crystallines, their aggregates and a lot of complete stones. These stone formation was markedly prevented by PUFS and SBST but the effectiveness of the former was superior to that of the latter when compared between two groups containing the same amount of soybean sterols (Table 6). It is easily reasoned that as this mouse model was made by loading the exogenous cholesterol, SBST which have been proved to inhibit competitively the intestinal cholesterol absorption may decrease cholesterol influx to the liver and therefore cholesterol efflux into bile and then as the result depress the gallstone formation, but the result that PUFS was more outstanding than SBST with regard to the prevention of stone production meant that the components in PUFS other than SBST might play an additive or a synergistic role. Details about this point are remained to be studied. In this experiment, the extent of decrease in serum and liver cholesterol by PUFS was parallel to the degree of the inhibition of gallstone formation, which indicated that among many hypolipidemic agents, PUFS was thought to be one of a few very interesting drugs with a desirable effect also on cholelithiasis.

In exp. II and III, the effect of PUFS on dissolution of gallstone once produced in gallbladder was investigated. Mice with gallstone prepared by feeding the lithogenic diet for 8 weeks were kept from 9th weeks on either normal diet or continuous lithogenic diet with or without PUFS. As shown in Table 7 and Table 8, PUFS had no effect on gallstone dissolution even 6 weeks later after exchanging diet but the related lipid metabolisms in serum and liver were clearly improved, among which a decrease in liver cholesterol was remarkable. As to this point, Tabata et al. reported that phytosterol given to rat increased bile acid and decreased cholesterol in bile[13-14]. Furthermore Begemann et al. found β-sitosterol to decrease the bile cholesterol contents in human[20]. From these facts it can be presumed that PUFS regulates cholesterol metabolism in direction for dissolving cholelith. Failure in dissolving gallstone by PUFS in this experiment may be due to the short time of treatment. Judging from the fact that there is no report so far in which the gallstone dissolution by any drug is exactly proved using a whole animal, it may be very difficult to investigate the gallstone dissolution in animal experiments.

Several investigators reported that CPIB, the most wide-spread hypolipidemic agent, rose the bile cholesterol level by increasing the cholesterol excretion from liver to bile with a consequence of deterioration of cholelithiasis[1,3,21-24] and other report showed that cholestyramine which inhibited the intestinal cholesterol uptake by absorption of bile acids essential to cholesterol absorption decreased the bile acid pool to lower cholesterol solubility in bile, making gallstone disease worse[25]. Recently chenodeoxycholic acid has rapidly come into the limelight as a new gallstone dissolution agent[26-30] but it has little effects on hyperlipidemia[31]. There have been reported only a few drugs to have good influences both on hyperlipidemia and cholelithiasis. As mentioned above, it was shown first and seems very worthy of note that PUFS already used as a clinical hypolipidemic medicine had a desirable efficacy for cholelithiasis and its potency was superior than phytosterol alone, which might promice to widen the merits of its clinical use.

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